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## Analysis of hepatitis C virus genotypes by a line probe assay and correlation with antibody profiles

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The 5' untranslated regions derived from 54 patients with a chronic hepatitis C virus infection were analyzed to determine the (sub)type of hepatitis C virus. Labelled polymerase chain reaction products from 5' untranslated region were used as probes for reverse hybridization in a line probe assay (Inno-LiPA) and results were validated by comparison with direct sequencing data. Five different genotypes could be distinguished based on 5' untranslated region sequence diversity. Results of typing by line probe assay and direct sequencing were similar. Antibody responses against core, NS-3, NS-4 and NS-5 epitopes were detected by RIBA-4 and Inno-LIA HCVAb II confirmatory assays. There was no consistent correlation between genotype and anti-HCV responses, although types 2, 3 and 4 hepatitis C virus isolates show poor reactivity with NS-4 epitopes. © Journal of Hepatology.

**Key words:** Direct sequencing; Line immuno assay; Polymerase chain reaction; Reverse hybridization; RIBA

Hepatitis C virus (HCV), the main etiological agent of post-transfusion hepatitis, is a small enveloped virus, which contains a positive sense, single-stranded RNA genome of approximately 9400 nucleotides (1). Based on genomic (2) and physico-chemical characteristics (3) of the virus, HCV is classified as a distinct member of the flaviviridae. From worldwide HCV isolates, several full length (4-9) and numerous partial sequences (10-18) have been obtained. Based on sequence diversity, several proposals were made to classify different HCV isolates (10,16,19-21), but there is no consensus in HCV nomenclature so far. A useful HCV classification was proposed recently (22), based on phylogenetic trees determined by Chan and colleagues (10), differentiating between types (approx. 68% average sequence homology) as well as between subtypes (approx. 79% sequence homology). Homologies between isolates belonging to the same subtype usually exceed 90%. This system was further extended by a new type, provisionally designated as type 4 (23).

There are indications that infections caused by different

HCV (sub)types may have different clinical implications (19). The effectiveness of antiviral treatment (24-26), efficiency of viral transmission, distribution among various patient populations and the development of hepatocellular carcinoma may also be subtype-related. Preliminary results urge HCV subtyping which complements the routine diagnostic antibody and reverse transcriptase-polymerase chain reaction (RT-PCR) assays.

All available sequence data were used to develop type- and subtype-specific probes for a reverse hybridization line probe assay, which has recently been described in detail (22). The assay is based on the observation that variation within the 5' untranslated region (UTR) is mainly restricted to two short (sub)type specific sequence motifs.

In this study HCV isolates were analyzed from 54 patients by the new line probe assay which allows classification of isolates into genotypes 1, 2, 3 (10,22), and 4 (23). Results of reverse hybridization were compared with data from direct sequencing of the 5' UTR. Furthermore, HCV antibody profiles, determined by recombinant im-

munoblot assay (RIBA)-4 and HCVAb II, were compared with the genotyping results.

## Materials and Methods

### Patient sera

Blood samples from 54 patients were obtained by venipuncture. Ethylenediaminetetraacetic acid (EDTA)-plasma was prepared within 2 h after collection, aliquotted, quickly frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . All patients had chronic HCV infection with elevated alanine aminotransferase (ALT) levels, biopsy-proven liver abnormalities and were anti-HCV and HCV-RNA positive (27).

### RNA isolation, and reverse transcriptase PCR

HCV-RNA was isolated from freshly frozen plasma samples by a modified version of the acid guanidinium-phenol-chloroform method as described (27). Briefly, cDNA was synthesized using antisense primer HCV19 (pos. -1 to -20; 5'-GTGCACGGTCTACGAGAC CT-3') and amplified by PCR using HCV19 and sense-primer HCV18 (pos. -323 to -304; 5'-GGCGA-CACTCCACCATAGAT-3') or HCV 35 (pos. -318 to -296; TTGGCGGCCGCACTCCACCATGAATGAC-TCCCC). PCR was performed for 40 cycles, consisting of 1 min  $94^{\circ}\text{C}$ , 1 min  $55^{\circ}\text{C}$ , and 1 min  $72^{\circ}\text{C}$ . Amplification products were analyzed by agarose gel electrophoresis and Southern blot hybridization using probe HCV17 (pos. -88 to -69; 5'-GAGTAGTGTGGGGTCGCGAA-3').

### Line probe assay

Based on all available 5' untranslated region sequences, several type-specific sequence motifs were recognized (see also 22). Motif 1 is located between positions -170 and -155, and motif 2 between -132 and -117. These motifs already allow discrimination between the different genotypes, are partially complementary and could possibly form a stable dsRNA stem structure (28). A number of positions displaying more subtle, but consistent variations allow consolidation of typing by means of motif 1 and 2, or enable more detailed subtyping.

The line probe assay (HCV line probe assay prototype version, Innogenetics, Ghent, Belgium) is based on the hybridization of labelled PCR amplification products to specific oligonucleotides directed against the variable regions of the 5' UTR. These probes were immobilized as parallel lines on membrane strips (reverse hybridization principle). During nested PCR, the product is biotinylated, which allows detection of hybrids by alkaline phosphatase labelled streptavidin. The HCV line probe assay

(Fig. 1) contains 15 probe lines, exposing 18 different 16-mer probes. Sixteen probes specifically recognize HCV genotypes, and two (no. 21 and 22) are general HCV probes (the location of the probes in the 5' UTR sequence is shown in Fig. 2). The development of the line probe assay was recently described in detail (22).

From the first-round PCR,  $0.5\ \mu\text{l}$  product was transferred into a new,  $50\ \mu\text{l}$  nested PCR reaction, containing primers HC3 (sense: -264 to -238: 5'-TCTAGCC-ATGGCGTTAGTRYGAGTGT-3'), HC4 (antisense: -29 to -54: 5'-CACTCGCAAGCACCTATCAGG-CAGT-3') and biotinylated  $^{11}\text{dUTP}$ . The line probe assay was performed according to the manufacturer's instructions. Briefly, biotinylated DNA was denatured by mixing  $10\text{--}20\ \mu\text{l}$  of the nested PCR reaction with NaOH and hybridized to the probes on the line probe assay in the presence of tetramethylammoniumchloride. After stringent washing, hybridization was detected by alkaline-phosphatase-conjugated streptavidin and substrate.

The results of the line probe assay were determined by scoring the presence or absence of hybridization with each probe line.

### Direct sequencing

PCR products were reamplified using sense primer NCR3 (pos. -314 to -288; 5'-GGGGCGGCCGCCA-CCATARRATCACTCCCCTGTGAGG-3'; underlined sequence is non-HCV specific) and LD58 (pos. -66 to -35; 5'-Bio-GGCCGGGGCGGCCGCCAAGCAC-CCTATCAGGCAGTACCACAAGGC-3') carrying a 5' biotin moiety. Biotinylated nested PCR products were used as a template for direct sequencing, using the protocol suggested by the manufacturer of the Dynabeads (Dyna, Norway). Briefly, nested PCR products were mixed with streptavidin-coated paramagnetic particles (Dynabeads M280, Dynal, Norway) in a binding and washing buffer ( $1\times\ \text{B\&W}$  buffer is 10 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA), to allow the binding of the biotinylated DNA. Complementary strands were separated by addition of NaOH, and sequenced using the T7 DNA sequencing kit (Pharmacia) and  $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$  (Amersham, Buckinghamshire, UK). DNA attached to the beads or in supernatant was sequenced with either NCR3 as a sense primer on the captured minus-DNA strand or NCR4 (pos. -66 to -47; 5'-CACTCTCGAGCACC-CTATCAGGCAGTACC-3') as an antisense primer on the plus strand in the supernatant.

### Anti-HCV assays

Antibodies to HCV were assayed by an enzyme immunoassay (EIA; Abbott Chicago, IL, USA) and confirmed by RIBA-4 (Ortho Diagnostics, Raritan, NJ,

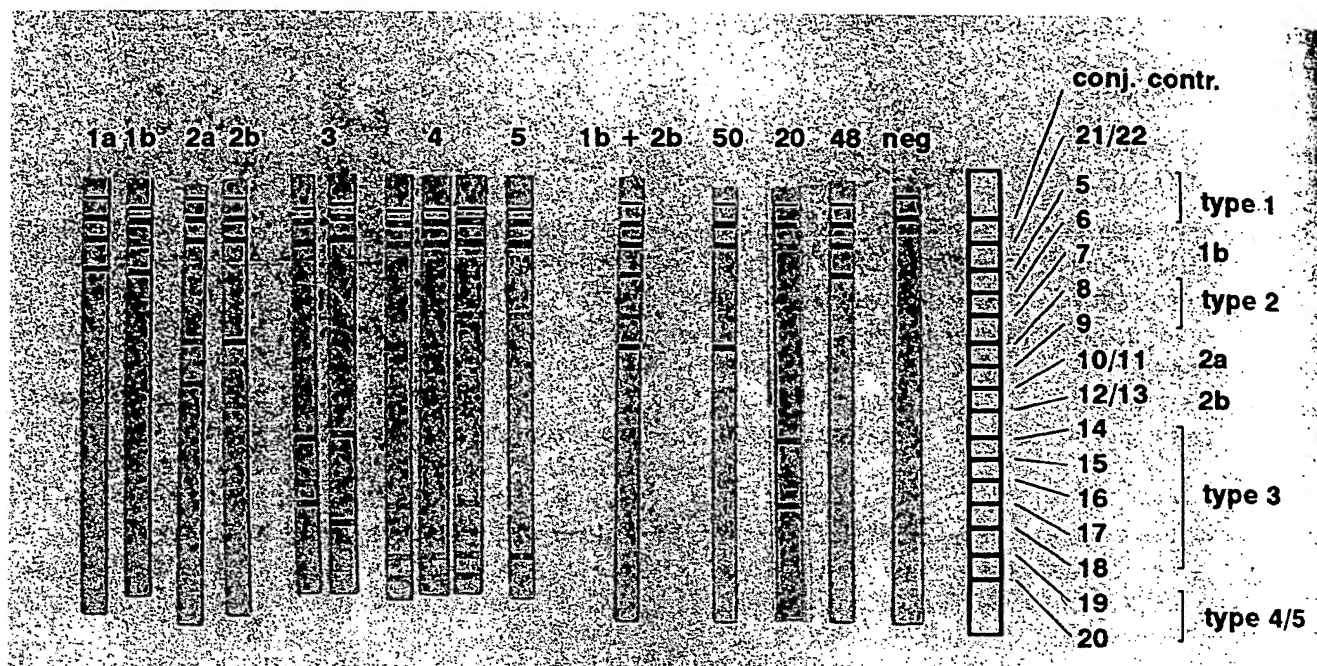


Fig. 1. The positions of the probe lines on the line probe assay strip. Probes 21 and 22 are general probes to identify general 5' UTR sequences. Probes 5-20 distinguish different types and subtypes of HCV as indicated. Representative results of isolates containing types 1a, 1b, 2a, 2b, and 3 are shown, together with all type 4 isolates. One isolate is provisionally classified as type 5. Furthermore, isolates 50 and 20, displaying sequence-specific variation and the single discrepant isolate 48, are shown.

USA) and Inno-line immunoassay (LIA) HCV Ab II (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions.

## Results

HCV-RNA was isolated from 54 patients with chronic HCV infection, and amplified by RT-PCR using 5' UTR-specific primers. These PCR products were analyzed by direct sequencing as described elsewhere (29). Biotinylated amplification products from nested reactions were used as target to the probes in the reverse hybridization line probe assay system. The location of the probes on the line probe assay strip and typical line probe assay results are shown in Fig. 1.

All labelled nested PCR products hybridized to general probes 21/22 and with a subset of genotype-specific probes. Results of line probe assay analysis of 54 patient isolates are summarized in Table 1. Four different genotypes were detected and there were no isolates that could not be typed by line probe assay. Observed hybridization patterns were consistent and classification into types and subtypes was obvious for all isolates, including one double infection with subtypes 1b and 2b. One isolate contained the SA1 sequence. This sequence can be classified provisionally as type 5 (Dr. P. Simmonds personal communication, 23).

Probes 17 and 18 were aimed to discriminate further between type 3 isolates, based on the presence of a G or A at -139, respectively. Most type 1 isolates cross-reacted with probe 17, and to a lesser extent with probe 18. Overall background staining was very low or completely absent.

Reverse hybridization results from line probe assay were compared with data obtained from direct sequencing. Sequences were determined on nested products derived from the same first-round PCR products as used for line probe assay. Sequences and location of line probe assay probes are shown in Fig. 2. Comparison of line probe assay and direct sequencing results revealed a small discrepancy in only one sample. Amplified HCV-cDNA from this isolate (no. 48, containing the DK7 sequence Fig. 2) hybridized with probe 7 (Fig. 1), indicating the presence of a G at pos. -99, but direct sequencing showed only an A. This isolate is therefore classified as 1b by line probe assay but as 1a by direct sequencing. On the other hand, some aberrant hybridization patterns observed in line probe assay were validated by direct sequencing results. Isolate 50 (containing sequence HC2-N2) failed to hybridize with probe 9. This is in full agreement with the presence of a G at -127. Isolate 20 (containing sequence HC3-N2) hybridized only very weakly to type 3-specific probe 15, which can be explained by the presence of a C at -118. The presence of these mutations

TABLE 1

Genotypes of 54 patient HCV isolates determined by the line probe assay

Type	No. of isolates	%
1a	7	13
1b	24	44
2a	7	13
2b	1	2
3	10	18
4	3	7
5	1	2
1b+2b	1	2

in regions used for typing never complicated the classification because 2 or 3 type-specific probes are present in the line probe assay. Probe 19 contains a C/T degeneracy at -167, which allows hybridization to all type 4 sequences obtained in this study, including SA1. Probe 20 does not hybridize to the SA1 sequence. In a number of isolates additional sequence variation outside the defined motifs 1 and 2 was detected by direct sequencing, but this did not affect the classification by the LiPA.

Antibodies against various HCV epitopes were assayed by RIBA-4 and LIA II. Results are shown in Table 2. One patient was negative by RIBA and LIA. One was indeterminate by LIA, as only a weak anti-core reaction was detected. From the single anti-HCV-negative sample, weak HCV-RNA signals were obtained. However, a sample from this patient, obtained 1 month earlier, was anti-HCV positive in RIBA. Only one patient specifically lacked core antibodies in both confirmatory assays; all other anti-HCV positive isolates were anti-core positive in both assays. All patients responded to NS-3 as determined by RIBA.

Type 2- and type 3-infected patients showed a low response rate to type 1 NS-4 epitopes, in both RIBA and LIA. NS-5 antibodies were detected by LIA in the majority of patients. The only double infection isolate did not contain antibodies against NS-4 epitopes.

### Discussion

This study describes the use of a new reverse hybridization line probe assay to determine the (sub)type of HCV in 54 well-characterized patient plasma isolates. The system was evaluated by direct sequencing and results are markedly similar.

There are several advantages in using the 5' UTR of the HCV RNA genome for genotyping. First, the 5' UTR is generally used in diagnostic PCR assays with universal primers to detect viremia. Therefore it is convenient to perform subsequent typing analysis on the resulting DNA product. Secondly, the sequence variations within the 5' UTR are limited to specific regions, such as motifs 1 and 2, located between highly conserved flanking sequences. This allows the use of general PCR primers as well as (sub)type-specific probes. This relatively high degree of conservation in the 5' UTR omits the necessity of (sub)type-specific primers to classify HCV using more variable coding regions of the genome (19). A large number of 5' UTR sequences have been published so far (10,22,30). Discrimination between types has been described using amplified cDNA derived from the 5' UTR for restriction fragment length polymorphism (RFLP, 23,31,32). Furthermore, the overall mutation rate of HCV has been estimated at approximately  $1.5 \times 10^{-3}$  base substitutions per site per year (12,33), but mutations occur unevenly along the genome. A hypervariable region located at the N-ter-

TABLE 2

Anti-HCV profiles of 54 patient isolates determined by RIBA-4 and Inno-HCV LIA HCVAbs II

Type	No. of patient	RIBA-4				Total	Inno-LIA			
		5-1-1 (NS4)	C100-3	C33c (NS3)	C22-3 (core)		NS-4	NS-5	Core	Total
1a	8	8	8	8	8	8	8	5	8	8
1b	23	22	19	22	22	22	22	13	22	22
2a	7	1	2	7	7	7	2	5	6	6
2b	1	0	0	1	1	1	0	1	1	1
3	10	0	4	10	10	10	2	7	10	10
4	3	0	1	3	3	3	2	2	3	3
5	1	1	1	1	1	1	1	1	1	1
1+2	1	0	0	1	1	1	0	1	1	1

Anti-HCV detection by RIBA and LIA. 5-1-1 and C100-3 are derived from the NS-4 region. C33c contains NS-3 and C22-3 covers core epitopes. Numbers in columns represent samples with a positive reaction against the specified epitope.

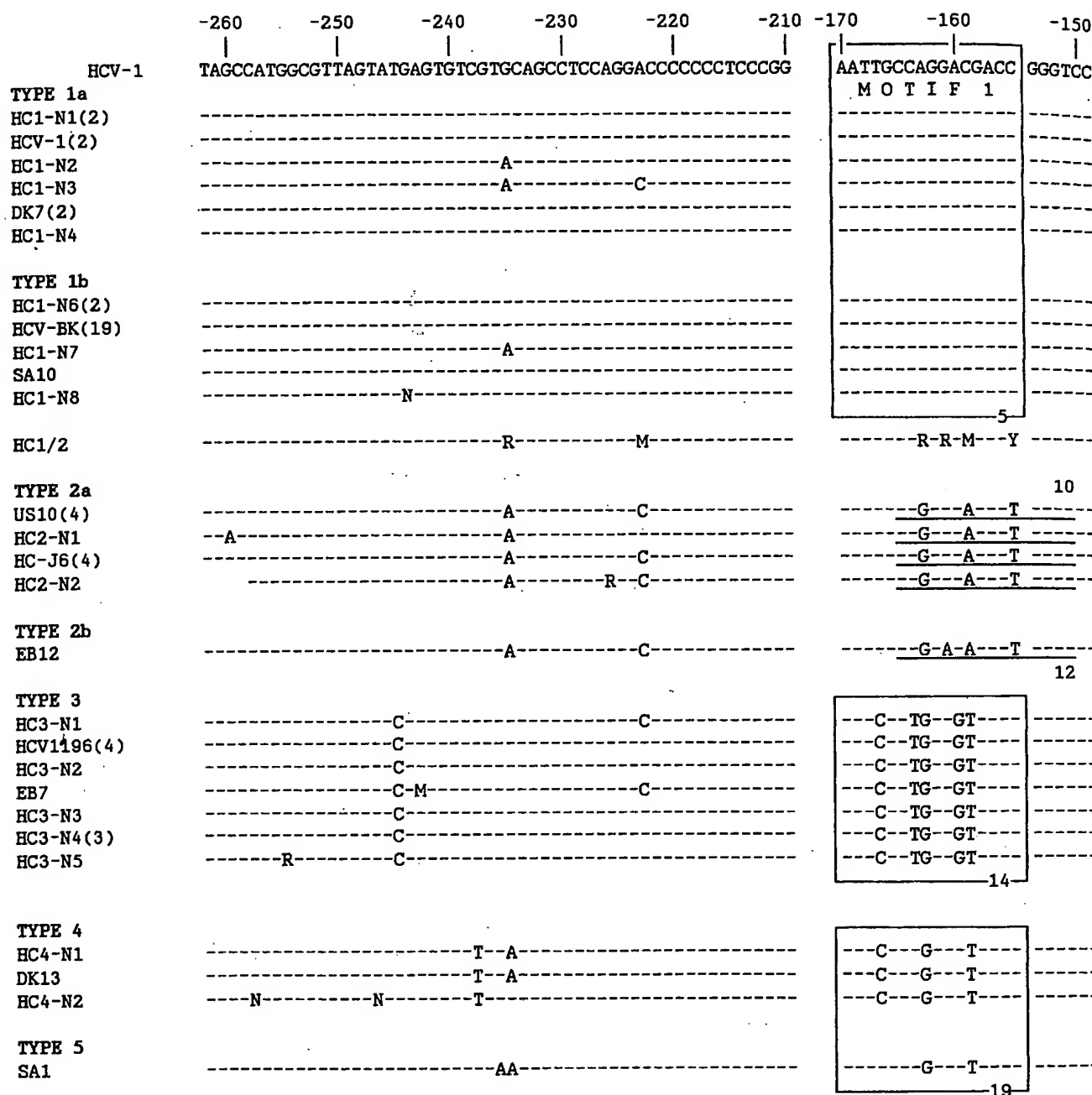
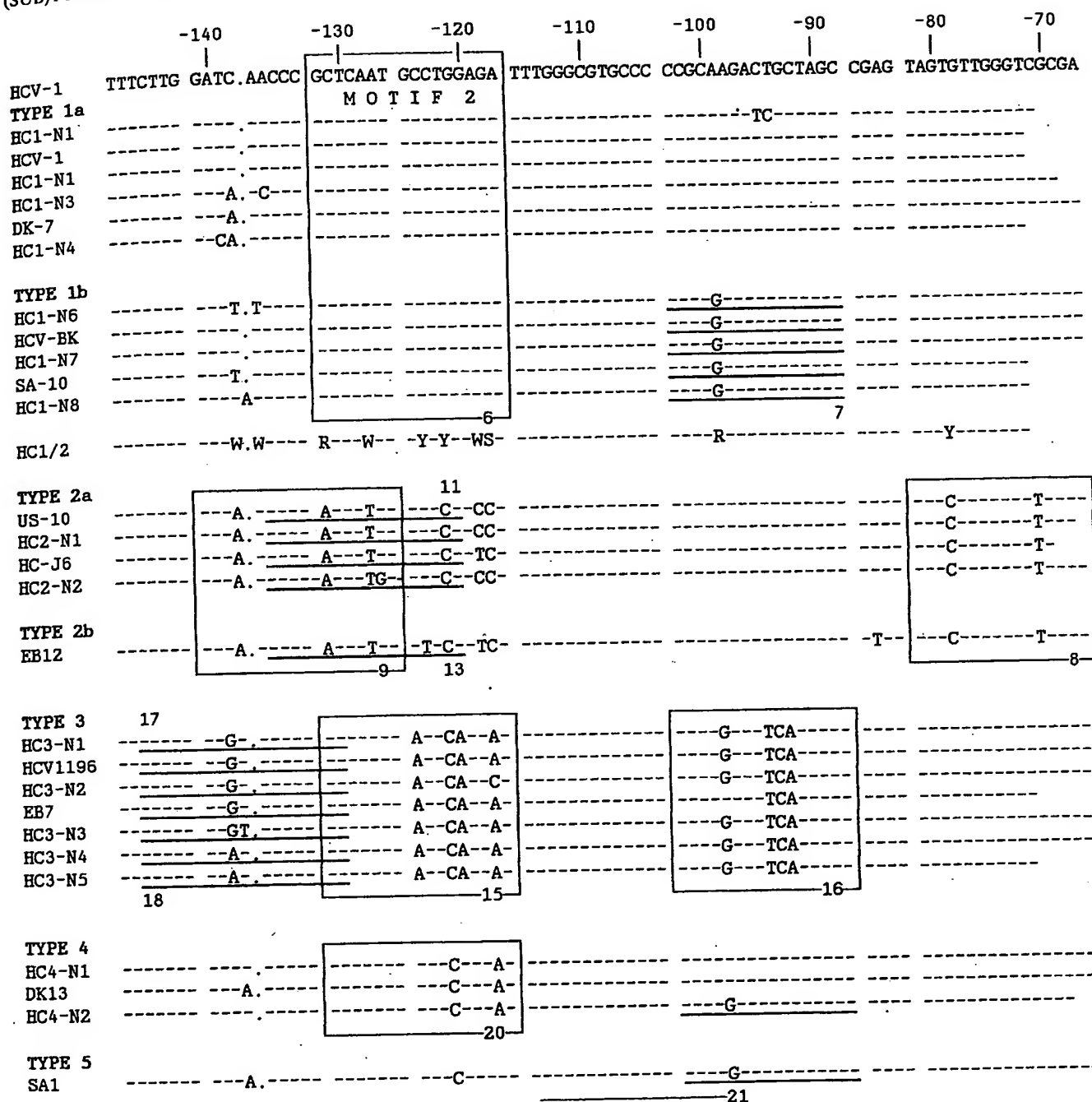


Fig. 2. Alignment of 5' UTR sequences (pos. -265 to -68) and positions of line probe assay (LiPA) probes. Sequences between pos. -210 and -170 are completely conserved and omitted from the figure. Sequences are grouped into five different genotypes. HCV-1 is the prototype sequence (6). DK-7, SA10, US10, and DK13 (30), HC-J6 (7, 8), EB-7 and EB-12 (10), and HCV1196 (18) were previously reported and identified by their original name. The positions of the LiPA probes 5-20 are identified by boxes (type-specific) or are underlined (subtype-specific). General probe 21 is underlined and probe 22 (pos. -178 to -194) is not shown in this figure. The SA1 sequence is tentatively classified as type 5.

minus of E2/NS1 has a high mutation rate, whereas the 5' UTR has a very low rate. Studies on genetic drift of HCV for more than 8 years in a chronically infected chimpanzee (33) and over 13 years in a chronic patient (12) showed a complete conservation of the 5' UTR. Finally, the putative secondary structure of the 5' UTR (23,28,34) implies functional conservation with respect to initiation of translation of the single open reading frame and ge-

nome replication (35). Therefore, there is probably high selective pressure on the function of this region. This is indicated by the existence of paired mutations (co-variants) in the complementary strands in the stem of the putative RNA-hairpin. This covariance, observed among different genotypes, conserves the secondary structure of motifs 1 and 2 (23). Finally, extensive sequence comparisons have shown (10) that sequence heterogeneity in the 5'



UTR displays similar phylogenetic relationships between HCV genotypes as in other regions such as core, NS3 and NS5. Therefore, sequence analysis of the 5' UTR should allow consistent discrimination of HCV types.

HCV RNA isolates from 54 chronic HCV patients were genotyped by 5' UTR analysis. Amplified cDNA from the 5' UTR was biotin-labelled during nested PCR and used as a probe for reverse hybridization in the line probe assay. Reverse hybridization offers a fast method of screening for the presence of specific sequences in a PCR product. The probes used in the line probe assay described here cover a considerable part of the entire 5' UTR se-

quence. The line probe assay contains both general and (sub)type specific probes and therefore allows detection of known as well as unknown HCV genotypes. Each of the 54 isolates described here hybridized to the general probes and could be further (sub)typed by line probe assay. New HCV types will fail to hybridize with the current (sub)typing probes on the strip, but will hybridize to the general probe. Aberrant hybridization patterns can also be observed on the line probe assay, as shown in isolates 20 and 50 (Fig. 2). Therefore the line probe assay provides an instrument for rapid identification of new HCV types or subtypes.



To evaluate the efficiency of the line probe assay system, the presence or absence of hybridization to each probe was compared with the corresponding results from direct sequencing. Single nucleotide differences were efficiently detected, e.g. to discriminate between type 2a and 2b by probes 10, 11, 12, and 13. In addition to published sequences, new variations were also detected by the line probe assay and confirmed by direct sequencing, as observed in isolates containing sequences HC3-N2 and HC2-N2. There was only one discrepancy between line probe assay and direct sequencing with isolate 48, although it was typed correctly as type 1 by the line probe assay. The question remains whether this isolate contains 1a or 1b sequences. A possible explanation is a coinfection with types 1a and 1b. A large excess of 1a sequences over 1b sequences could explain the failure of direct sequencing to detect both A and G at -99 and the weak hybridization of the PCR product with probe 7. The directly obtained sequence represents the major sequence present in an isolate. Furthermore the line probe assay identifies subtype 1a based on absence of hybridization with probe 7. Positive identification of each subtype could further improve the reliability of the line probe assay. Probes 17 and 18 cross-reacted with most of the type 1 isolates. The reason for this is unclear. The value of probes 17 and 18 in discriminating between the presence of a G or A at position -139, is doubtful. The target sequence for these probes is located in the region forming a single stranded RNA loop in the putative secondary structure of the genomic RNA (28). This might also explain the relatively high frequency of mutations detected in this region, including the insert in HC1-N8. Four isolates could not be classified as genotype 1, 2 or 3. Three of those are classified as type 4 and one (containing sequence SA1) is tentatively designated as type 5. More data from these isolates are necessary to justify this provisional classification.

One double infection with subtypes 1b and 2b was detected (Fig. 1 and 2; isolate HC1/2) by both line probe assay and direct sequencing. It is much easier, both to detect and to (sub)type the double infection by line probe assay than by direct sequencing. Interpretation of double signals at one nucleotide position by direct sequencing can be difficult and may require advanced experience in reading sequence autoradiographs.

Although the version of the line probe assay described here did not yet contain type 5 specific probes, it was possible to discriminate between type 4 and 5 sequences because probe 19 contains a degeneracy. Addition of type 5 specific probes onto the line probe assay strip will further facilitate recognition of this genotype.

Profiles of antibodies against specific HCV epitopes were determined. Two confirmatory immunoblot assays,

capable of detecting antibodies against epitopes from core, NS-3, NS-4 and NS-5, could not discriminate between different genotypes. RIBA-4 uses expression products of recombinant cDNA clones, derived from various parts of the HCV genome, whereas the Inno-LIA exposes a number of synthetic peptides. At present, as many more sequences of genotypes 1, 2, 3, and 4 became available, significant sequence heterogeneity in various parts of the genome (10, 21) may be observed. Consequently, infection with other HCV genotypes may evoke antibodies against type-specific epitopes, which are not optimally recognized by type 1 epitopes in the RIBA and LIA. This is illustrated by the absence of antibodies against NS4 epitopes in most type 2 and 3 isolates (36, Table 2). Antibodies against clone 5-1-1 are even completely absent in type 3 isolates. The single indeterminate LIA results on an isolate classified as type 2a may be partly explained by genotype-specific immune reactions.

Antibodies against core and NS-5 epitopes show a higher degree of cross-reactivity. It was possible to distinguish a number of type 1 and 2 isolates by assaying anti-core antibodies, directed against two different type-specific core peptides (37), although specificity was limited. Type-specific serological assays would considerably facilitate discrimination between different HCV genotypes. Sequence information on antigenic regions is still limited. Therefore, many more sequence data from all different genotypes must be obtained, to develop (sub)type-specific antigens for antibody assays. However, there are considerable differences in immune reactivity among individual patients. In some patients, antibody responses are poor; e.g. in isolate 23, antibodies against core were undetectable. Also, in immune-compromised patients serological testing is difficult, due to lack of sufficient antibody titers. These conditions prevent serological HCV typing.

In summary, sequence analysis of the 5' UTR of HCV allows consistent genotyping of all presently known HCV isolates. Furthermore, reverse hybridization systems in a strip test format like the described line probe assay, provide fast and reliable typing of HCV isolates and identification of new HCV types. This assay allows the use of the amplification products from 5' UTR after RT-PCR assays and therefore conveniently complements the routine HCV diagnosis. Screening of large patient populations is feasible, and could lead to rapid identification of new HCV genotypes.

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